Glycerol-Mediated Repression of Glucose Metabolism and Glycerol Kinase as the Sole Route of Glycerol Catabolism in the Haloarchaeon *Haloferax volcanii*†

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Although glycerol is the primary carbon source available to halophilic heterotrophic communities, little is known regarding haloarchaeal glycerol metabolism. In this study, a gene encoding a glycerol kinase homolog (*glpK; HVO_1541*) was deleted from the genome of the haloarchaeon *Haloferax volcanii* by a markerless knockout strategy. The *glpK* mutant, KS4, readily grew on yeast extract-peptone complex medium and glucose minimal medium but was incapable of growth on glycerol. Glycerol kinase activity was dependent on the *glpK* gene and readily detected in cells grown on glucose and/or glycerol, with the activity level higher in medium supplemented with glycerol (with or without glucose) than in medium with glucose alone. An analysis of carbon utilization revealed that glycerol suppressed the metabolism of glucose in both the parent H26 and *glpK* mutant strains, with catabolite repression more pronounced in the glycerol kinase mutant. Transcripts specific for *glpK* and an upstream gene, *gpdA*, encoding a homolog of glycerol-3-phosphate dehydrogenase subunit A, were upregulated (8- and 74-fold, respectively) in the presence of glycerol and glucose compared to those in the presence of glucose alone. Furthermore, *glpK* was transcriptionally linked to the *gpdC* gene of the putative glycerol-3-phosphate dehydrogenase operon (*gpdABC*), based on the findings of reverse transcriptase PCR analysis. The results presented here provide genetic and biochemical evidence that glycerol metabolism proceeds through a glycerol kinase encoded by *glpK* and suggest that a glycerol-3-phosphate dehydrogenase encoded by the upstream *gpdABC* operon is also involved in this pathway. Furthermore, our findings reveal a unique example of glycerol-induced repression of glucose metabolism in *H. volcanii*.

Halophilic and halotolerant microorganisms have adapted different methods for withstanding the high osmotic pressure exerted by their surrounding hypersaline environment. Halophilic archaea (7, 13), as well as the halophilic bacterium *Salinibacter ruber* (19), maintain a high intracellular salt concentration by accumulating K\(^+\) and Cl\(^-\) ions and excluding Na\(^+\) ions, thus requiring intracellular proteins to be active under high-salt conditions. Many halophilic bacteria (24), the halotolerant green alga *Dunaliella* sp. (3), and some haloarchaea (12) exclude cytoplasmic salts and rely on organic solutes such as ectoine, glycine betaine, and glycerol to provide osmotic balance. Glycerol, in particular, is accumulated in molecular form, where it serves as a primary energy source for haloarchaea. Upon uptake, halophilic microorganisms assimilate glycerol into dihydroxyacetone phosphate (DHAP) by one of two catabolic routes (Fig. 1). In one route, glycerol is first phosphorylated by glycerol kinase to form sn-glycerol-3-phosphate, which is subsequently oxidized by sn-glycerol-3-phosphate dehydrogenase to produce DHAP. Alternatively, glycerol can be first oxidized by glycerol dehydrogenase to form dihydroxyacetone (DHA), which is subsequently phosphorylated by an ATP- or phosphoenolpyruvate:phosphotransferase system-dependent DHA kinase to yield DHAP. Once generated from glycerol, DHAP can be channeled into pyruvate and other metabolic intermediates, including sn-glycerol-1-phosphate, used as a phospholipid backbone in archaea (16).

Although glycerol is an important carbon and energy source for members of halophilic, heterotrophic communities, little is known regarding glycerol metabolism, especially in haloarchaea. Halophilic archaea such as *Haloferax volcanii* have been demonstrated previously to metabolize glycerol (22), and specific activities of glycerol-metabolizing enzymes in various haloarchaea have been determined (18, 22, 25); however, the metabolic pathways surrounding glycerol utilization at the molecular level have not been described. Here, we provide genetic and biochemical evidence that *H. volcanii* metabolizes glycerol via a glycerol kinase (encoded by *glpK*) and, most likely, a *glpK*-linked sn-glycerol-3-phosphate dehydrogenase (encoded by a *gpdABC* operon). We also present data suggesting that glycerol may serve as a catabolite repressor of glucose metabolism in *H. volcanii*. Our results provide insight into the central metabolic pathways of heterotrophic haloarchaea such as *H. volcanii*.

**MATERIALS AND METHODS**

**Materials.** Biochemicals and α-glycerophosphate dehydrogenase used for glycerol kinase activity analyses were purchased from Sigma-Aldrich (St. Louis, MO). Other organic and inorganic analytical-grade chemicals were from Fisher Scientific (Atlanta, GA) and Bio-Rad (Hercules, CA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IN). dUTP coupled to digoxigenin (DIG) by an 11-atom spacer, alkaline phosphatase-conjugated anti-
body raised against DIG, disodium 3-(4-methoxy-3,2-dihydro-3-oxo-1H-[1,2]dioxetane-3,2-dione-4yl) phenyl phosphate (CSPD), and other DIG-related biochemicals were from Roche Molecular Biochemicals (Indianapolis, IN). Positively charged membranes for Southern hybridization were from Ambion (Austin, TX). Phusion and Taq DNA polymerases, restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs (Ipswich, MA). Standard agarose used for the separation of DNA for Southern blotting and routine analysis was from Bio-Rad. Hi-Lo DNA standards were from Minnesota Molecular, Inc. (Minneapolis).

Strains, media, and plasmids. Strains, oligonucleotide primers used for cloning, and plasmids are summarized in Table 1 and Table S1 in the supplemental material. Escherichia coli DH5α was used for routine recombinant DNA experiments. *H. volcanii* strains were transformed (8) using plasmid DNA isolated from *E. coli* GM2163. Liquid cultures were aerated at 200 rpm. *E. coli* strains were grown at 37°C in Luria-Bertani medium supplemented with 100 mg per liter ampicillin as needed. *H. volcanii* strains were grown at 42°C in various media, including yeast extract-peptone-Casamino Acids, Casamino Acids, glycerol minimal medium (Gly MM), Gly MM supplemented with glucose (Gly Glu MM), and glucose minimal medium (Glu MM). Medium formulae were according to The Halohandbook (9), with the following exception: 20 mM glycerol and/or glucose served as the sole carbon source(s) for the minimal medium. Media were supplemented as needed with novobiocin (0.1 μg · ml⁻¹), 5-fluoroorotic acid (50 μg · ml⁻¹), and uracil (10 and 50 μg · ml⁻¹ for growth in the presence and absence of 5-fluoroorotic acid, respectively). Uracil and 5-fluoroorotic acid were solubilized in 100% dimethyl sulfoxide at 50 mg · ml⁻¹ prior to addition to the growth medium.

For growth assays, cells were grown in yeast extract-peptone-Casamino Acids, Gly MM, Gly Glu MM, and Glu MM as indicated below. Cells from −80°C glycerol stocks were freshly inoculated onto the appropriate agar-based media on plates. Cells were thrice subcultured and used as inocula for the final analyses of growth under various conditions as described below. Each subculture was inoculated to a final optical density at 600 nm (OD₆₀₀) of 0.03 to 0.04. For the analyses of growth rates and cell yields, cells were grown in 20 ml of medium in 250-ml baffled Erlenmeyer flasks. For enzyme activity assays, cells were grown in 100 ml of Gly MM, Glu MM, or Gly Glu MM in 1,000-ml flasks. For RNA preparation, cells were grown in 3 ml of medium in 13- by 100-cm² culture tubes. Cell growth was monitored by an increase in OD₆₀₀ (where 1 OD₆₀₀ unit equals approximately 10⁹ CFU · ml⁻¹ for all strains used in this study). All experiments were performed at least in triplicate.

**DNA isolation and analysis.** DNA was separated by electrophoresis using 0.8% (wt/vol) agarose gels in 1× TAE electrophoresis buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5). Plasmid DNA was isolated from *E. coli* strains by using the QiAprep spin miniprep kit (Qiagen, Valencia, CA). PCR products were purified by using the MinElute kit (Qiagen) prior to modification by a restriction enzyme (BamHI, HindIII, KpnI, XbaI, HpaI, or NdeI) or T4 DNA polynucleotide kinase. For rapid PCR screening, template DNA was extracted from *H. volcanii* mutant and parent strains and recombinant *E. coli* DH5α as
with 3 M KCl, and lysed by sonication (four pulses of 20 s at 140 W). Debris was removed by centrifugation (10 min at 12,000 g and 4°C), and the protein concentration in the cell extract was estimated using the Bradford assay with bovine serum albumin as a standard.

Glycerol kinase (ATP:glycerol-3-phosphotransferase; EC 2.7.1.30) specific activity was assayed by a coupled photometric reaction as described previously (23), with the following modifications. The reaction mixture (5 ml) contained 0.5 ml of cell extract (0.5 to 1 mg of protein · ml⁻¹), 30 μM ATP, and 100 μM l-cysteine in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 3 M KCl. The reaction (at 42°C to parallel cell growth) was started by the addition of 125 μmol glycerol. Negative control mixtures lacked ATP or glycerol, or the reactions were performed using boiled cell lysate from the parent strain H26. Samples (0.45 ml) were withdrawn at 10, 20, 30, 40, 45, 50, 60, and 90 min after the start of the reaction, which was terminated by the addition of 0.45 ml of 0.2 N H₂PO₄, and the reaction mixture was used to measure the formation of glycerol-3-phosphate with rabbit muscle glycerol-3-phosphate dehydrogenase as described previously (23).

DNA sequencing. The fidelity of all PCR-amplified products was confirmed by sequencing both DNA strands of the plasmid inserts listed in Table 1, as well as those of the mutant strains, by Sanger automated DNA sequencing using an Applied Biosystems model 3130 genetic analyzer (ICBR Genomics Division, University of Florida).

Southern blotting. Genomic DNA isolated from H. volcanii parent and mutant KS4 strains was subjected to digestion with SphiI and BspHI and analyzed by CSPD-mediated chemiluminescence using DIG-labeled probes specific for the chromosomal region cloned into H. volcanii cells (29, 30). Genomic DNA was isolated from 5-ml cultures by DNA spooling (9).

A gene (gfk; HV0_1541) encoding a glycerol kinase homolog was targeted for markerless deletion from the chromosome of H. volcanii H26 by using a gpk2-based “pop-in/pop-out” method (2, 5). Colonies were screened for the absence of a readily generated 500-bp PCR product by using internal primers (Negative-Forward and Negative-Reverse primer pairs) specific for the coding region of the gene. Strains which did not generate this PCR product were confirmed to be mutant strains by Southern blotting (as described above) and PCR with Confirm-Forward and Confirm-Reverse primer pairs, which anneal to a site outside the H. volcanii genomic region cloned into the suicide plasmid (pJAM2675). The latter PCR products were separated by agarose gel electrophoresis and sequenced to confirm DNA fidelity (as described above). The sequences of primers used for the cloning and screening of mutant colonies are provided in Table S1 in the supplemental material.

Chromosomal gene knockout. A gene (gfk; HV0_1541) encoding a glycerol kinase homolog was targeted for markerless deletion from the chromosome of H. volcanii H26 by using a gpk2-based “pop-in/pop-out” method (2, 5). Colonies were screened for the absence of a readily generated 500-bp PCR product by using internal primers (Negative-Forward and Negative-Reverse primer pairs) specific for the coding region of the gene. Strains which did not generate this PCR product were confirmed to be mutant strains by Southern blotting (as described above) and PCR with Confirm-Forward and Confirm-Reverse primer pairs, which anneal to a site outside the H. volcanii genomic region cloned into the suicide plasmid (pJAM2675). The latter PCR products were separated by agarose gel electrophoresis and sequenced to confirm DNA fidelity (as described above). The sequences of primers used for the cloning and screening of mutant colonies are provided in Table S1 in the supplemental material.

**Enzyme activity assays.** All experiments were performed in biological triplicate, and the means ± standard deviations (SD) of the results were calculated.

**Exponential-growth-phase cells** were harvested by centrifugation (15 min at 6,000 × g and 4°C), resuspended in 100 mM potassium phosphate buffer (pH 7.4) with 3 M KCl, and lysed by sonication (four pulses of 20 s at 140 W). Debris was removed by centrifugation (10 min at 12,000 g and 4°C), and the protein concentration in the cell extract was estimated using the Bradford assay with bovine serum albumin as a standard.

**Glycerol kinase (ATP:glycerol-3-phosphotransferase)**: EC 2.7.1.30 specific activity was assayed by a coupled photometric reaction as described previously (23), with the following modifications. The reaction mixture (5 ml) contained 0.5 ml of cell extract (0.5 to 1 mg of protein · ml⁻¹), 30 μM ATP, and 100 μM l-cysteine in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 3 M KCl. The reaction (at 42°C to parallel cell growth) was started by the addition of 125 μmol glycerol. Negative control mixtures lacked ATP or glycerol, or the reactions were performed using boiled cell lysate from the parent strain H26. Samples (0.45 ml) were withdrawn at 10, 20, 30, 40, 45, 50, 60, and 90 min after the start of the reaction, which was terminated by the addition of 0.45 ml of 0.2 N H₂PO₄, and the reaction mixture was used to measure the formation of glycerol-3-phosphate with rabbit muscle glycerol-3-phosphate dehydrogenase as described previously (23).

**DNA purification.** Total RNA was isolated from H. volcanii parent H26 cells (exponential phase; OD₆₀₀ 0.4 to 0.6) by using RNeasy RNA purification columns (Qiagen). RNA was treated with amplification-grade DNase I according to the recommendations of the supplier (Sigma-Aldrich), with the following modifications: 3 U of enzyme per μg of RNA was added, and the mixture was incubated for 15 min at 22°C. The integrity of RNA was determined by agarose gel electrophoresis. The RNA concentration was determined by assessing A₂₆₀ with a Bio-Rad SmartSpec 3000 instrument.

**qRT-PCR and RT-PCR.** Quantitative reverse transcriptase PCR (qRT-PCR) and RT-PCR were performed using H. volcanii total RNA (0.1 μg) as the template, appropriate primers (see Table S1 in the supplemental material), iQSYBR green supermix (Bio-Rad), and an iCycler (Bio-Rad). RNA was reverse transcribed to generate cDNA by using an iSCRIPT kit according to the instructions of the manufacturer (Bio-Rad). After cDNA synthesis (at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min), qRT-PCR mixtures were preheated to 95°C (4 min) and subjected to 40 amplification cycles consisting of denaturation (95°C for 30 s), annealing (1 min at temperatures listed in Table S1 in the supplemental material), and elongation (72°C for 17 s). Final extension was performed at 72°C (10 min). For RT-PCR, reaction mixtures were preheated to 95°C (4 min) and subjected to 40 amplification cycles consisting of denaturation (95°C for 30 s), annealing (58°C for 1 min), and elongation (72°C for 41 s). For each primer pair, negative and positive controls were included to exclude genomic DNA contamination and confirm primer pair function, respectively. For the controls, reactions were identical, with the following exceptions: the negative control sample was maintained on ice during the reverse transcription step, and for the positive control, H. volcanii genomic RNA was used as a template.

**HPLC.** At various time points during growth on Glu MM and Gly Glu MM, 1-ml samples of both parent (H26) and glycerol kinase gfk mutant (KS4) cultures were withdrawn and centrifuged (10 min at 10,000 g and 4°C).

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**Table 1. List of strains and plasmids used in this study**

**Strain or plasmid** | **Description** | **Source or reference**
--- | --- | ---
DH5α | F· recA1 endA1 hsdR17(k- mK­1 M­1) supE44 thi-1 gyrA relA1 | Life Technologies
GM2163 | F· ara-14 leuB6 thiA1 lacY1 tss78 glvV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL130 dam13-Tn5 xylA5 mil-1 thi-1 mcrB1 hsdR2 | New England Biolabs

**H. volcanii strains**

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<tr>
<td>H26</td>
<td>Wild-type isolate</td>
<td>2</td>
</tr>
<tr>
<td>KS4</td>
<td>H26 gfk (devoid of GfK)</td>
<td>This study</td>
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**Plasmids**

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<td>pJAM20502</td>
<td>Ap·, Nv·, control plasmid derived from pBAP010</td>
<td>G. Y. Zhou, unpublished data</td>
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<td>pJAM2055</td>
<td>Ap·, Nv·, pJAM2052-derived expression plasmid including Hpal site for C-terminal fusion to StreptII tag</td>
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<tr>
<td>pJAM2658</td>
<td>Ap·, pTA131-derived suppressive plasmid containing gfk with −500 bp genomic DNA sequences flanking 5' and 3' ends of gfk</td>
<td>This study</td>
</tr>
<tr>
<td>pJAM2675</td>
<td>Ap·, pJAM2658-derived gfk suicide plasmid</td>
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<tr>
<td>pJAM2666</td>
<td>Ap·, Nv·, pJAM2055-derived expression plasmid containing P_mn gfk-StreptII tag</td>
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*The StreptII tag is a peptide that binds to the biotin binding site of streptavidin. Ap·, ampicillin resistance; Nv·, novobiocin resistance.*
RESULTS AND DISCUSSION

Glycerol is metabolized through glycerol kinase. To analyze glycerol catabolism in *H. volcanii*, a gene encoding a glycerol kinase homolog (HVO_1541; glpK) was targeted for knockout in a *pyrE2* mutant strain (H26). The deduced product of this gene, GlpK, was most closely related (with 74 to 78% identity) to and clustered in dendrograms with other putative glycerol kinases of haloarchaea, including those of *Halorubrum lacusprofundi*, *Haloarcula marismortui*, *Haloquadratum walsbyi*, *Natrionalba magadii*, and *Halobacterium salinarum* (sp. NRC-1), with the notable absence of GlpK homologs in the haloalkaliphilic archaeon *Natronomonas pharaonis* and other archaea (see Fig. S1 in the supplemental material). The *H. volcanii* and other haloarchaeal GlpK proteins also clustered with the bacterial glycerol kinases with the greatest degrees of identity (up to 58%) to those of the “Thermoaerobacteriales” and *Thermotogales* (see Fig. S1 in the supplemental material).

The GlpK gene was deleted from the chromosome of *H. volcanii* by a markerless knockout strategy as described previously (2, 5). Gene deletion was confirmed by PCR, Southern blotting, and sequencing analysis (Table 1; see also Table S1 and Fig. S2 in the supplemental material). The resultant glycerol kinase mutant, H26 ΔglpK (KS4), was incapable of growth either on Gly MM plates (Fig. 2) or in liquid culture (data not shown). A pHV2-based self-replicating plasmid containing the *glpK* gene under the control of a strong rRNA P2 promoter (pJAM2666) complemented KS4 for growth on Gly MM, while the plasmid vector alone (pJAM202c) did not complement this *glpK* mutant (Fig. 2).

Based on these results, we conclude that the glycerol kinase homolog encoded by *glpK* is required for the growth of *H. volcanii* on glycerol. Previous studies reported glycerol kinase activity in the lysate of *H. volcanii* cells grown in the presence of glycerol, while glycerol dehydrogenase activity remained undetectable (e.g., reference 22). These early findings support our claim that in *H. volcanii*, glycerol metabolism proceeds through the *glpK*-encoded glycerol kinase rather than the conversion of glycerol to DHA by a glycerol dehydrogenase. Thus, our results also suggest that genes HVO_1546 to HVO_1544, which are predicted to encode putative DHA kinase subunits K, L, and M based on the *H. volcanii* genome sequence (communicated by J. Eisen, TIGR; http://archaea.ucsc.edu/, April 2007 version), function in DHA metabolism and not glycerol metabolism. Consistent with this possibility, recent evidence suggests that *Salinibacter ruber* mediates the incomplete oxidation of glycerol to yield DHA as an overflow product, which may then be taken up by different types of heterotrophs present in hypersaline environments (4).

To biochemically confirm that the *glpK* homolog HVO_1541 codes for glycerol kinase, the specific activities of glycerol kinase in cell lysates from H26 and KS4 (H26 ΔglpK) were measured as described previously (6, 20). In brief, the generation of glycerol-3-phosphate (the product of the phosphorylation reaction catalyzed by glycerol kinase) was evaluated by a photometric, coupled assay that included glycerol as a substrate. Glycerol-3-phosphate dehydrogenase, which catalyzes the NAD\(^+\)-dependent oxidation of glycerol-3-phosphate to form DHAP, was also included in the assay. The reduction of NAD\(^+\) to NADH was monitored at 410 nm, and product formation was quantified using standards of glycerol-3-phosphate. By using this assay, glycerol kinase activity was readily detected in cells of the parent strain H26 grown in medium containing glycerol (Gly MM or Gly Glu MM), regardless of the presence of glucose (Fig. 3). Significant levels of glycerol kinase activity were also detected in H26 cells grown on medium with glucose alone (Glu MM), although the levels were reduced about two-fold compared to those in H26 cells grown on media with glycerol (i.e., Gly MM and Gly Glu MM). Glycerol kinase activity was not detected in the glycerol kinase mutant (KS4) or boiled cell lysate (the negative control) (Fig. 3). The specific activity of glycerol kinase in parent strain H26 grown in the presence of glycerol was measured as 430 ± 30 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) (Fig. 3), which is about fivefold or more higher than previously reported specific activities of glycerol kinase proteins in *Salinibacter ruber* (90 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\)), *Halobacterium cutirubrum* (14 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\)), and *H. volcanii* (61 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\)) for cells grown in complex medium with peptides, determined in similar assays (20, 23, 25), this value was within a reasonable range of measurement for glycerol kinase enzymes.

Our results demonstrate that the *glpK* homolog HVO_1541 encodes a glycerol kinase and provide strong evidence that this gene is required for the catabolism of glycerol in *H. volcanii*. Note that glycerol kinase activity is not universal among the archaea, unlike bacteria, and is absent in those organisms that cannot use glycerol as an energy source (e.g., autotrophic methanogens [16]). Thus, glycerol kinase is not thought to be involved in the synthesis of the backbone of archaeal phospho-
lipids and is instead mediated by sn-glycerol-1-phosphate dehydrogenase, which generates sn-glycerol-1-phosphate from DHAP. Therefore, glycerol does not appear to be channeled into DHA by a glycerol dehydrogenase for the growth of \textit{H. volcanii}.

Our results also reveal that glycerol kinase activity in \textit{H. volcanii}, although stimulated by growth on glycerol, is not inhibited by the presence of glucose in the growth medium. The detection of comparable levels of glycerol kinase activity in \textit{H. volcanii} cells grown on Gly MM regardless of glucose supplementation directly contrasts with results for the \textit{E. coli} model, in which glucose exhibits catabolite repression of the \textit{glpK} regulon encoding glycerol kinase (10).

Glycerol serves as a catabolite repressor of glucose metabolism. To determine if the growth defect of the glycerol kinase mutant (KS4) was exclusive to glycerol and to further investigate the observed differences in catabolite repression between \textit{H. volcanii} and \textit{E. coli}, the growth of and utilization of carbon by the \textit{H. volcanii} H26 parent and KS4 mutant strains on minimal medium supplemented with either 20 mM glycerol and 20 mM glucose or 20 mM glucose alone (Gly Glu MM or Glu MM, respectively) were measured (Fig. 4 and 5). Although there are other carbon sources (i.e., Tris buffer, uracil, biotin, and thiamine) in the minimal medium, these alone do not support the growth of \textit{H. volcanii} (data not shown). Thus, any observed growth would be due to glucose and/or glycerol supplementation.

The \textit{glpK} mutant (KS4) and parent strain H26 grew and utilized glucose at nearly identical rates when cultured in minimal medium containing glucose as the sole carbon source (Glu MM) (Fig. 4). In both cases, roughly 85% of the glucose was metabolized within 60 h, contributing to an average overall

![Graph showing glycerol kinase activity](http://jb.asm.org/)

**FIG. 3.** \textit{H. volcanii} glycerol kinase activity is dependent on \textit{glpK} (HVO_1541), stimulated by growth on glycerol, and uninhibited by glucose supplementation. \textit{H. volcanii} H26 (parent) and KS4 (\textit{glpK} mutant) cells were grown in Gly Glu MM, Gly MM, or Glu MM. Measurements of glycerol kinase specific activities were performed using lysates prepared from log-phase cells as specified in Materials and Methods. * indicates that enzyme activity was not detected. Experiments were performed in biological triplicate, and the means ± SD were calculated.

![Graph showing growth and glucose utilization](http://jb.asm.org/)

**FIG. 4.** The parent strain H26 and glycerol kinase mutant KS4 (H26 \textit{Δglpk}) exhibit similar growth rates, cell yields, and carbon utilization patterns when grown in Glu MM. Growth at 42°C (200 rpm) was monitored by an increase in OD$_{600}$, where 1 U was equivalent to approximately $10^9$ CFU per ml for all strains. At various time points, supernatant fractions were withdrawn from both parent H26 and KS4 cultures and analyzed via HPLC for glucose consumption. Experiments were performed in triplicate, and the means ± SD were calculated. Cell growth and glucose utilization levels are indicated.
OD$_{600}$ of 1.27 and a growth rate of 0.11 doublings per h. The addition of glycerol to the Glu MM (producing Gly Glu MM) enhanced the growth rate and cell yield of the parent H26 approximately twofold, to 0.22 doublings per h and an OD$_{600}$ of 2.0 (Fig. 5). Consistent with these results, H26 metabolized both of these carbon/energy sources but displayed a preference for glycerol, with 79% of the glycerol and only 16% of the glucose in the Gly Glu MM being metabolized (Fig. 5). Thus, H26 metabolized glucose at a reduced rate when glycerol was included in the growth medium. In contrast to H26, the glpK mutant KS4 was unable to metabolize glycerol and did not induce glucose metabolism when grown in the presence of glycerol until approximately 30 h later than its parent, H26 (Fig. 5), and 20 h later than KS4 grown on medium with glucose alone (Fig. 4). Once KS4 initiated this delayed metabolism of glucose in glycerol-supplemented medium (Gly Glu MM), the growth rate (0.095 doublings per h) and final OD$_{600}$ (0.93) of these cells were only slightly reduced compared to those of cells grown in medium with glucose alone.

Based on these results, it appears that *H. volcanii* mediates glycerol-dependent catabolite repression of glucose metabolism. Glucose metabolism by both *H. volcanii* strains examined was repressed by the presence of glycerol, with the repression in the glpK mutant more pronounced than that in its parent, based on the extreme lag observed in the growth of KS4 on medium with glycerol and glucose compared to that on glucose alone. The observed preference of *H. volcanii* for glycerol directly contrasts with that of *E. coli*, which exhibits diauxic growth with glucose as the preferred carbon and energy source (11). Catabolite repression by carbon compounds other than glucose is not novel. For example, members of the genus *Pseudomonas* exhibit organic acid-induced catabolite repression of glucose metabolism (15). However, glycerol-induced catabolite repression has not been reported previously.

Levels of glycerol-3-phosphate dehydrogenase and glycerol kinase transcripts are upregulated by the addition of glycerol. Based on the genome sequence (Hartman et al., unpublished), *H. volcanii* has two putative NAD(P)-linked sn-glycerol-3-phosphate dehydrogenase operons: one (gpdA2B2C2; HVO\_A0269 to HVO\_A0271) on the minichromosome pHV4 and one (gpdABC; HVO\_1538 to HVO\_1540) on the chromosome directly upstream of the glycerol kinase gene (Fig. 6A). The protein paralogs encoded by these two operons are closely related in primary amino acid sequence (58 to 74% identical and 67 to 85% similar) and cluster with oxidoreductases such as the A, B, and C subunits of sn-glycerol-3-phosphate dehydrogenase. The products of both operons are distinct from the enantiomeric glycerophosphate synthase (EgsA), an NAD(P)-linked sn-glycerol-1-phosphate dehydrogenase responsible for the formation of the glycerol-1-phosphate backbone of archaeal phospholipids (16) and most likely encoded by HVO\_0822 in *H. volcanii*.

In order to determine whether either gpd operon is upregulated in the presence of glycerol, qRT-PCR was performed...
using gene-specific primers (see Table S1 in the supplemental material for all primer details). Primers were designed to correspond to the first gene (gpdA2 [HVO_A0269] and gpdA [HVO_1538]) in each of the two operons in order to achieve the strongest signal for transcriptional analysis (Fig. 6A). In addition, qRT-PCR primers for glpK were designed to determine if the transcription of this gene was induced by glycerol. A transcript specific to the ribosomal protein L10 gene (ribL) was used as an internal control based on data from previous studies (17) and our confirmation by qRT-PCR that the level of induction of transcripts specific for ribL in cells grown in the presence of glycerol and glucose was close to onefold compared to transcript levels in cells grown in the presence of glucose alone.

By qRT-PCR analysis, transcripts specific for glpK, gpdA, and gpdA2 were detected at significant levels under all growth conditions examined (growth on Gly MM and Gly Glu MM) (Fig. 6B). In addition, transcripts specific for gpdA and glpK were upregulated approximately 78- and 9-fold, respectively, in the presence of glycerol and glucose compared to those in the presence of glucose alone (Fig. 6B). In contrast, gpdA2 transcripts were not induced to significant levels (2.0-±1.0-fold)
by glycerol (Fig. 6 B), indicating that the gpdA2 operon is not likely to be involved in glycerol metabolism. Our results reveal that transcripts specific for glpK and its gene neighbor gpdA are significantly induced by glycerol, supporting the argument that both genes and their encoded enzymes (glycerol kinase and glycerol-3-phosphate dehydrogenase) are involved in glycerol metabolism in \textit{H. volcanii}.

Glycerol kinase and glycerol-3-phosphate dehydrogenase genes are under the control of a common promoter. Due to the close proximity of glpK and gpdABC and the likely involvement of the encoded gene products in a common metabolic pathway (14, 21), we investigated whether these genes were cotranscribed in an operon. RT-PCR was performed using primers designed such that the forward primer would anneal to the 3′ coding region of gpdC and the reverse primer would anneal to the 5′ coding region of glpK, amplifying a portion of each gene as well as the 364-bp intergenic region (Fig. 6A). A single PCR product of the expected size was detected using synthesized cDNA (Fig. 6C), and the sequence of the product was later confirmed. No product from the negative control reaction with RNA as a template was detected (Fig. 6C). Thus, glpK and gpdC are linked at the transcriptional level.

Although we have demonstrated that glpK and gpdC of the putative gpdABC operon are transcriptionally linked, the reasons for the significant differences in the levels of induction of glpK- and gpdA-specific transcripts in the presence of glycerol (Fig. 6B) remain to be determined. Multiple promoter elements may be involved in the transcription of this region of the chromosome and account for these differences in induction. Consistent with the possibility that multiple promoters control the expression of the gpdABC-glpK region, BRE and TATA box promoter consensus sequence elements were identified upstream of both gpdA and glpK (Fig. 6A, P1 and P2, respectively). Alternatively, the transcription of glpK (which is distal relative to gpdA) may be reduced by transcriptional polarity, which leads to the premature termination of polyistronic mRNA translation, resulting in the reduced transcription of genes located distally from the operon (27). Another possibility is that the glpK-specific transcripts are more susceptible to degradation than those specific for gpdA.

**Conclusion.** We demonstrate in this study that glycerol metabolism in \textit{H. volcanii} requires glycerol kinase encoded by the glpK gene (HVO_1541) and that this gene is transcriptionally linked to a putative glycerol-3-phosphate dehydrogenase operon (gpdABC; HVO_1538 to HVO_1540) located upstream of glpK on the chromosome. The levels of both glpK- and gpdA-specific transcripts are significantly upregulated in the presence of glycerol, although not to the same extent, with the glycerol-dependent induction of gpdA-specific transcripts being eightfold greater than that of glpK-specific transcripts. Promoter consensus elements upstream of both gpdA and glpK suggest that, in addition to sharing a common promoter with gpdA, glpK may be regulated independently of gpdA. Our present model is that glpK and gpdA share a common P1 promoter immediately upstream of gpdA that is tightly regulated in response to glycerol availability and that additional control of glpK transcription is achieved through a gpdA-independent P2 promoter immediately upstream of glpK.

In this study, we also provide evidence that glucose metabolism in \textit{H. volcanii} is under catabolite control by glycerol and speculate that this control is mediated by a GlpR-type regulator. Future phenotypic and biochemical characterization of a glpR-deficient strain is expected to provide insight into gene function in \textit{H. volcanii}, as well as other microorganisms with similar gene organization patterns. Overall, the results not only shed light on glycerol metabolism in \textit{H. volcanii}, but also add to our understanding of central metabolic pathways of haloarchaea.

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